$\phi_i^V$ vapour-phase fugacity coefficient for component *i*
$\gamma_i$ activity coefficient for component *i*

**See Colour Plate 5.**

## Further Reading

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Seader JD and Henley EJ (1998) *Separation Process Principles*. New York: John Wiley.

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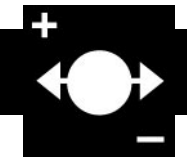
# ELECTROPHORESIS

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## An Outline of the Historical Background to Electrophoretic Separations

The movement of charged particles under the influence of an electric field was observed as long ago as 1807 by Ferdinand Frederic Reuss. In 1909, the term, electrophoresis, was introduced by Michaelis as a description of this phenomenon and is derived from the Greek word *elektron* meaning amber (i.e. electric) and *phore* meaning bearer. Yet it was not until the 1930s that electrophoresis, as we know it today, developed from the work of Tiselius, who, in 1948, was awarded the Nobel Prize for this development. **Table 1** charts the development of the technique over the last century. By the 1950s electrophoresis was a common laboratory technique equivalent in usefulness to planar chromatography techniques such as paper and thin layer. However, with the advent of high-performance liquid chromatography (HPLC) in the 1970s, analytical electrophoresis became something of a 'Cinderella' technique. Only in biochemical and clinical laboratories did electrophoresis continue in use as a qualitative separation technique for macromolecules, such as proteins and DNA.

It has been claimed that currently at least half of all separations are performed by electrophoresis since separations of blood proteins and DNA digests are routinely performed by the technique. The technique is now so routine in biomedicine and related disciplines that it is rarely referred to in the abstracts and titles of papers where it is a core technology, for example DNA sequencing. Even so, it is mentioned by name in almost as many papers as is chromatography (**Table 2**). However, with the development of capillary electrophoresis after 1981 electrophoresis has returned as a substantial topic of interest to mainstream analytical chemistry.

## Fundamentals of Electrophoresis

Unlike chromatography, there is no formal International Union of Pure and Applied Chemistry (IUPAC) definition of electrophoresis, although one is being developed at the time of writing. However, through teaching the subject over the past 10 years, I have developed the following definition:

> 'Electrophoresis is a mainly analytical method in which separations are based on the differing mobilities (i.e. speed plus direction of movement) of two or more charged analytes (ionic compounds) or particles held in a conducting medium under the influence of an applied direct current electric field' (**Figure 1**).

Electrophoresis therefore contrasts to chromatography which is defined as a method used primarily for the separation of two or more components of a mixture, in which the components are distributed between two phases, one of which is stationary while the other moves. Another difference is that in chromatography the modelling of the separation from first principles is complex, difficult and imprecise whereas a relatively simple theoretical background to electrophoresis has been developed and is reproduced below. For a more complete discussion of electrophoretic theory see Mosher *et al*. (1992).

In electrophoresis the movement is towards the electrode of opposite charge to that of the particle or ion being separated. Cations are positively charged ions and move towards the negative electrode (the cathode). Anions are negatively charged ions and move to the positive electrode (the anode). It is important to note that neutral species do not move under the influence of the electric field, although they

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**Table 1** An outline history of electrophoresis

| *Date* | *Researcher(s)* | *Development* |
|---|---|---|
| 1807 | Reuss | Observed movement of colloids in an electric field – the discovery of electrophoresis |
| 1886 | Lodge | $H^+$ migration in a tube of phenolpthalein 'jelly' (i.e. zone electrophoresis) |
| 1892 | Picton and Lindner | Invention of boundary electrophoresis |
| 1892 | Smirnow | Electrofractionation of diphtheria toxin solution |
| 1893 | Whetham | 'Moving boundary' separation of solution of coloured ions in vertical tubes |
| 1899 | Hardy | Globulin movement in U-tube with electric current |
| 1904 | Romer | Diphtheria toxin solution separation via U-tubes |
| 1905 | Hardy | Detailed study of globulins with various U-tube designs |
| 1907 | Field and Teague | Toxin/antitoxin separated via agar tube bridges between beakers of sample and water |
| 1912 | Ikeda and Suzuki | Isoelectric focusing (IEF) first observed during studies on amino acid electrophoresis |
| 1914 | Schwerin | Patent describing electrophoresis of colloidal systems |
| 1923 | Kendall and Crittenden | Preparative separation of isotopes in agar U-tube |
| 1930 | Tiselius | Moving boundary studies of proteins in solution |
| 1937 | Tiselius | Improved apparatus for moving boundary studies including ultraviolet detection of proteins |
| 1939 | Coolidge | Electrophoretic separation of serum proteins in tubes of glass wool |
| 1946 | Consden *et al.* | 'Ionophoresis' of amino acids and peptides in silica gel slab; first 'blotting' experiments |
| 1948 | Wieland and Fischer | Filter paper electrophoresis |
| 1950 | Hagland and Tiselius | Electrophoresis in a glass powder column |
| 1952 | Tiselius | Electrophoresis in filter paper and other media |
| 1954 | Kolin | Introduced artificial pH gradients for IEF |
| 1954 | Strain and Sato | Introduction of 'electrochromatography' |
| 1956 | Porath | Column electrophoresis using cellulose powder |
| 1959 | Raymond and Weintraub | Introduced polyacrylamide gels (PAGE) |
| 1964 | Ornstein and Davis | Design of apparatus for tube 'disc' electrophoresis |
| 1965 | Tiselius *et al.* | 'Free zone' electrophoresis of virus particles in 3 mm internal diameter rotating capillary |
| 1965 | Hjerten *et al.* | 'Particle sieving' electrophoresis of ribosomes in polyacrylamide gel electrophoresis (PAGE) tube gels |
| 1965 + | Many continued developments in microcapillary tube gel electrophoresis and isotachophoresis | |
| 1967 | Shapiro *et al.* | Sodium dodecyl sulfate (SDS)/PAGE technique for the determination of the molecular weight of proteins |
| 1975 | O'Farrell and Klose | Independently introduced two-dimensional IEF/SDS–PAGE |
| 1979 | Mikkers *et al.* | High-performance electrophoresis in polymer capillaries |
| 1981 | Jörgenson and Lukacs | Theoretical/expermental studies on high-resolution electrophoresis in glass capillaries (CZE) |
| 1984 | Terabe | Micellar electrokinetic capillary chromatography (MEKC) introduced |
| 1987 | Microphoretics | First commercial capillary electrophoresis system |

may diffuse from the load position or be carried by electroosmotic flow.

The rate of migration (velocity) of any charged particle in an electric field can, at its simplest, be considered to be the vector sum of a driving force (the electrical force) and any resisting or aiding forces. Any ion, compound or body carrying an overall charge at a given pH value will move in solution

**Table 2** Separation techniques in biomedicine and related areas

| Separation mode | Type | Total number of papers |
|---|---|---|
| Electrophoresis | All modes | 150 000 |
| | Simple planar | 13 800 |
| | Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) | 78 000 |
| | Immuno | 19 700 |
| | Isoelectric focusing | 14 400 |
| | Pulsed field | 1 600 |
| | Two-dimensional PAGE | 4 800 |
| | Capillary electrophoresis | 6 700 |
| Chromatography | All modes | 250 000 |
| | Planar | 52 000 |
| | Column | 98 000 |
| | High-performance liquid chromatography | 62 500 |
| | Gas chromatography | 32 000 |

Papers in Medline and on the author's database from 1976 to 1999.

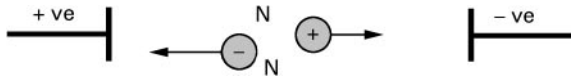


**Figure 1** The basic principle of all electrophoretic separations is that charged ions attempt to move in an electric field towards the electrode of opposite polarity. Neutral compounds do not move.

under appropriate conditions. In simple solutions, ions will move freely toward the electrode of opposite charge and the product of the charge on the ion and the applied electric field ($E$) gives the electric force experienced by the ion. However, since even a simple ion can be considered as a particle this movement is opposed by a frictional drag given by Stokes' law.

The driving force is represented by the potential gradient along which the charged particle moves and is given by the electric field strength ($E$):

$$E = \frac{\text{applied voltage}}{\text{distance between electrodes}} = \frac{V}{D} \qquad [1]$$

The applied field ($F_{ef}$):

$$F_{ef} = qE \qquad [2]$$

where $q$ is the total charge on the ion.

The friction ($F_{fr}$) drag is given by:

$$F_{fr} = 6\pi r\upsilon\eta \qquad [3]$$

where $\eta$ is the viscosity of the media, $r$ is the 'radius' of the molecule and $\upsilon$ is the velocity.

On applying a voltage there is a rapid acceleration of all the molecules and equilibrium is achieved in a few microseconds. A vector diagram at equilibrium for movement in an electric field is shown in **Figure 2**, and under such circumstances the following equilibrium conditions apply:

- at equilibrium:

$$F_{ef} = F_{fr} \qquad [4]$$

- therefore:

$$qE = 6\pi r\upsilon\eta \qquad [5]$$

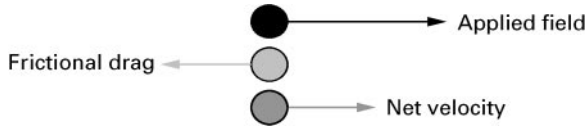


**Figure 2** A vector diagram for movement in an electric field at equilibrium.

- so the velocity is given by:

$$\upsilon = \frac{qE}{6\pi r\eta} \qquad [6]$$

Mobility ($\mu$) is defined as the average velocity with which an ion moves under unit applied electric field under defined conditions:

$$\mu = \frac{\text{average migration velocity}}{\text{electric field strength}} = \frac{\upsilon}{E} \qquad [7]$$

Substituting for $\upsilon$ from eqn [3]:

$$\mu = \frac{q}{6\pi r\eta} \qquad [8]$$

Mobility can therefore be interpreted as proportional to a charge-to-size ratio for a molecule in a given buffer at a set pH. Absolute mobility is the apparent or measured mobility corrected by any other effects adding to or subtracting from the absolute mobility (an example is electroosmotic drift). Sometimes, the term relative mobility is used when the apparent mobility of one compound is calculated in relation to another compound.

The units of $\mu$ are cm $s^{-1}$ divided by V $cm^{-1}$ and are therefore $cm^2\,V^{-1}\,s^{-1}$. The magnitude of $\mu$ for typical small ions is of the order of $10^{-6}$ $cm^2\,V^{-1}\,s^{-1}$, e.g. for the sodium ion, $\mu = 5 \times 10^{-6}$ $cm^2\,V^{-1}\,s^{-1}$ or for the protein albumin, $\mu = -1.5 \times 10^{-4}$ $cm^2\,V^{-1}\,s^{-1}$. Note the difference in the sign of the mobility and hence the direction of movement.

A small, highly charged species, for example, an ion, will have a high mobility and a large ionizable compound, such as a protein, will have a low mobility. It is clear that electrophoresis can readily separate large and small ionic species. Then, for two or more closely related ionic species, if the basic descriptors used in eqn [6] are known, it should be a simple matter to calculate the separation conditions and the degree of resolution that can be achieved. The charge on an ionizable molecule, such as an amino acid, is determined by the pH of the electrophoretic medium. The viscosity of the medium at a given temperature will also be known precisely. The difficulty from a theoretical predictive standpoint is to determine the radius of a molecule. Many attempts have been made to calculate this value from first principles and these are discussed later. It is also clear that the distance moved is proportional to the applied voltage and the time period over which the voltage is applied.

The above analysis of the movement is a further simplification in that the resisting forces are not just

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due to simple frictional drag but can also include effects such as:

- size and shape of the molecule;
- electrolyte concentration;
- solubility;
- adsorption to surfaces;
- complexation with species in the electrolyte solution.

The two basic electrical equations that govern electrophoresis are first Ohm's law which states that $V = iR$, where $i$ is the current and $R$ is the resistance. Secondly, power, that is the product of current and voltage or, alternatively, the heat generated is given by $i^2R = W$ where $W$ is power measured in watts. This heating effect is termed Joule heating. The quantity of heat generated is, of course, time dependent. The development of heat and then temperature gradients in electrophoresis leads to:

- convection currents;
- diffusional/thermal broadening;
- evaporation;
- viscosity changes;
- pH variations;
- thermal degradation of analytes especially proteins and matrices.

These changes all result in band broadening and the lower resolution of separated analytes. Heat can also lead to gels drying, buffers boiling and in extreme situations even fires.

The effect of heating processes on electrophoretic separations is complex and often variable. Traditionally, workers have countered these effects by using buffers that generate low currents, for example dilute buffer solutions, low conductivity buffers, by operating at low voltages (100–500 V), operating in constant current or constant power modes and, of course, using external cooling systems, such as circulating cold water around the instrument.

### Electroendosmosis

The second important effect in electrophoresis is electroosmotic flow (EOF) or electroendosmosis. If ionized groups cannot migrate in an electrical field (e.g. when part of a static support medium) then the liquid adjacent must move in order to maintain thermodynamic equilibrium. This EOF is seen as a bulk movement of liquid over a solid surface. In the case of silica or the carboxyl residues on a sheet of paper, the water will move in the direction of the cathode with a characteristic mobility ($\mu_{eof}$) depending on pH. The $\mu_{eof}$ is related to the zeta-potential of the solid surface and the dielectric constant of the electrolyte. As with mobility of an ion, $\mu_{eof}$ also varies with the viscosity of the electrolyte. The magnitude of the EOF is pH dependent. EOF leads to diffusional broadening of separated bands in slab electrophoresis but is used to good effect in capillary electrophoresis (see later section).

## The Modes of Electrophoresis

There are four fundamental modes of electrophoresis, at least, in the traditional planar formats, namely moving boundary electrophoresis, zone electrophoresis, isoelectric focusing and isotachophoresis.

### Moving Boundary Electrophoresis

This was the method that was studied by Tiselius in his detailed development of electrophoresis.

In this system the sample plus buffer are placed in the appropriate sample reservoir (electrolyte chamber). Tiselius used a U-tube containing the sample mixed with the buffer and the electrodes were dipped into each end. A single buffer solution therefore connects the two electrodes by way of the separation matrix. When the potential is applied, the analytes in the sample separate according to their differing mobilities. In the situation shown in **Figure 3** the cation of highest mobility moves ahead of the rest such that its front is resolved from the others. However, only the fastest analyte and, possibly, the slowest are truly separated and then only partly. The resolution of the components from others then diminishes. Tiselius observed the separated analytes via changes in their refractive index using Schlieren optics. Today such a separation mode is of little practical use except possibly in a preparative mode.

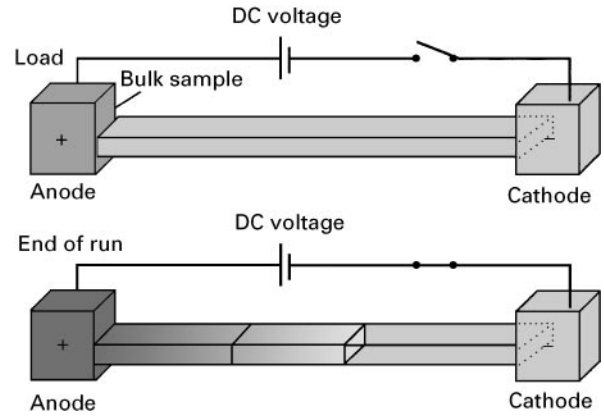


**Figure 3** (See Colour Plate 6) The principle of moving boundary electrophoresis.

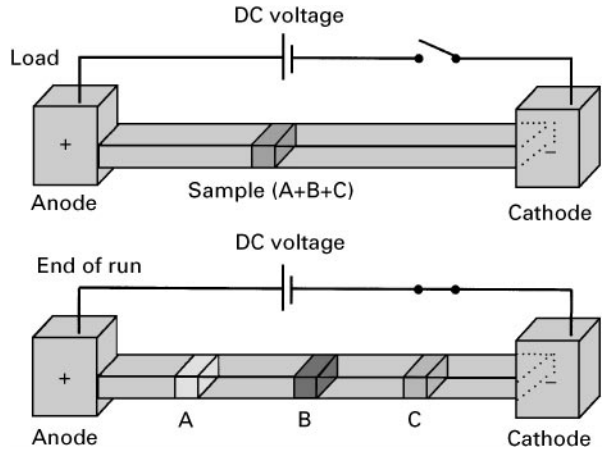


**Figure 4** (See Colour Plate 7) The principle of zone electrophoresis.

### Zone Electrophoresis

This is probably the most common form of electrophoresis in both slab and capillary formats (see **Figure 4**). The sample is loaded as a discrete plug or zone into a buffered electrolyte filled media such as a gel. The load point is somewhere between the electrodes in the middle if the charge on the analytes is not known or nearer to one than the other if the ionic nature of the analytes is known. The two electrode chambers and the matrix contain the same buffer. On application of the voltage, which in traditional systems is 100–500 V DC, the components of the sample migrate at different speeds and possibly in differing directions due to their differing mobilities and their respective charges. So, after an appropriate time period, they will have separated into (hopefully) distinct zones. Turning off the voltage terminates the separation. The degree of separation will depend on the voltage applied, the distance over which the sample separates and the time as well as the nature of the analytes and the buffer. The resultant zones will most often be of a distribution that is broader than the original loading zone due to the thermal mixing and so on that will have occurred during the separation process. With few exceptions some means of revealing the zones such as staining will be necessary.

The major factor controlling the mobility of the analytes in zone electrophoresis is the pH of the buffering electrolyte. For most ionic species the buffer controls the degree of ionization via the relationship expressed in the following equation (Henderson–Hasselbach equation):

$$\begin{aligned} \mathrm{pH} &= \mathrm{p}K_\mathrm{a} + \log(1/\alpha - 1) \text{ for anions,} \\ &\quad -\log(1/\alpha - 1) \text{ for cations} \end{aligned} \qquad [9]$$

For analytes such as peptides or proteins which contain a number of independently ionizable groups, the overall charge on the molecule is given by summing the contribution from each group at the pH in question:

$$\mu_{\mathrm{eff}} = \sum \alpha_i m_i \qquad [10]$$

where $m_i = \mu_i$ of each completely ionized form and $\alpha_i$ is the degree of ionization of each ionized form. An example of the change in charge for a number of different peptides is shown in **Figure 5**.

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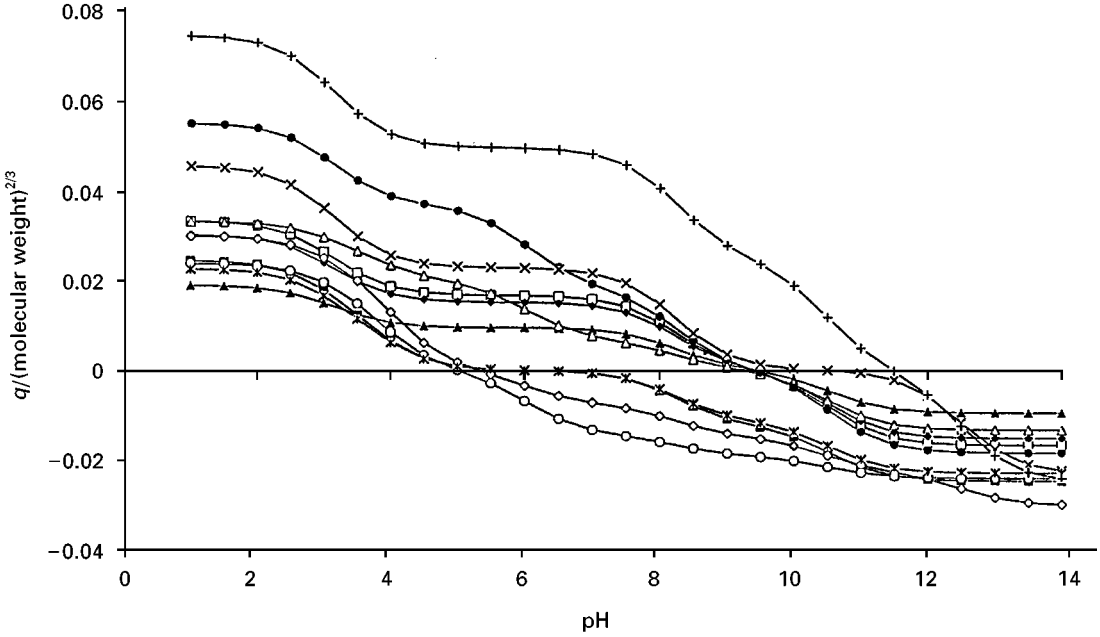


**Figure 5** An example of the change in charge for a number of different peptides. ■, AT1; □, AT2; ◆, AT3; ◇, AT4; ▲, AT5, △, AT6; ●, AT7; ○, AT9; ×, AT10; ⁎, AT11; |, AT14.

Since the mobility of an ion is a charge-to-size ratio (eqn [8]), one way to modify this for macromolecules is to change the size and shape of the molecules. For proteins this may be brought about by configuration changes due to changes in the pH, and in the case of DNA by incorporating an intercalating agent such as ethidium bromide. It is less simple to change the size and shape of small molecules, but one possible technique is complexation. For example, sugars will complex with borate ions in borate buffers and metal ions can complex with both peptides and nucleotides.

Equation [8] also indicates that mobility is dependent on the frictional drag exerted by the background electrolyte; in other words its viscosity. Adding agents such as polyethylene glycol (PEG) or cellulose derivatives to the buffer can increase viscosity and hence change the separation achieved.

### Isoelectric Focusing

Isoelectric focusing (IEF) is steady-state electrophoresis in a pH gradient. Ionic compounds migrate to the point in the gradient where they have zero overall charge and therefore zero net mobility. For proteins this means that they migrate to their isoelectric point (PI value). The pH gradients used can be linear or nonlinear and cover either wide pH ranges, for example, 2–10 or narrow ranges ( $< 2$ pH units). The pH gradient is formed by the application of a voltage to a mixture of amphoteric compounds (ampholytes) with closely spaced pIs encompassing a chosen pH range. A mix of at least 200 individual ampholyte species is required to establish a pH gradient of 4–10. Typically, the protein mixture is mixed with ampholytes and the resultant mixture is placed between the electrodes (**Figure 6**). The synthesis of such carrier ampholytes by attaching carboxylic acid moieties to polyvalent amines was perfected by Vesterberg in the Karolinska Institute in 1964. Even the first application showed the remarkable resolving power of IEF when two myoglobin molecules differing in pI by only 0.05 pH units were resolved.

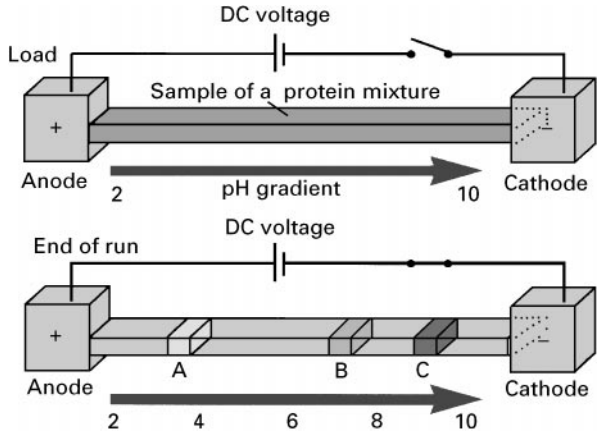


**Figure 6** (See Colour Plate 8) The principle of isoelectric focusing.

The resolving power of IEF is very high and is described by the following equation:

$$\Delta \mathrm{pI} = \left( \frac{D(\mathrm{dpH}/\mathrm{d}x)}{-E(\mathrm{d}\mu/\mathrm{dpH})} \right) \qquad [11]$$

where $D$ is the diffusion coefficient, $E$ is the electric field strength and $\mu$ is the mobility of the proteins. Good resolution between analytes is therefore favoured first by low diffusion coefficients (i.e. ideal for proteins) and a steep mobility profile through their pI, and secondly by high field strengths and a shallow pH gradient.

Once a protein has reached its pI position in the pH gradient, effects such as diffusion and EOF will cause some drift from the focused zone. This drift is countered by the fact that the protein will develop a small amount of ionization and migrate back to its pI position in the pH gradient and so become refocused. Focusing takes time, depending on the voltage used and the nature of the analytes: electrophoresis may need several hours before a steady-state is reached. Proteins are therefore resolved into narrow concentrated bands. In fact this concentrating effect is such that solubility problems can occur.

IEF was commercialized by LKB and the carrier ampholytes marketed under the tradename of Ampholine. The use of polyacrylamide gels was introduced to simplify the IEF methodologies. Although commercialized, the early ampholyte systems were in simple solution but separations tended to be irreproducible due to drifting towards the electrodes due to EOF effects. Modern IEF uses immobilized ampholytes that were developed by Righetti and coworkers in 1982. In such systems the ampholytes are immobilized to a gel matrix usually contained in a thin cylinder and so giving stable pH gradients in an easy to use format. The resolving power of these new gels with immobilized pH gradients (IPG) was much improved and permitted resolution to 0.001 pH units. More recent systems, such as the Pharmacia IPGphore, use ampholytes immobilized on flexible plastic strips. Once focused, the strips can be easily transferred to a second dimension or even used as the target in mass spectrometry.

### Isotachophoresis

Isotachophoresis (ITP) is a form of steady-state electrophoresis in which a voltage gradient generated by

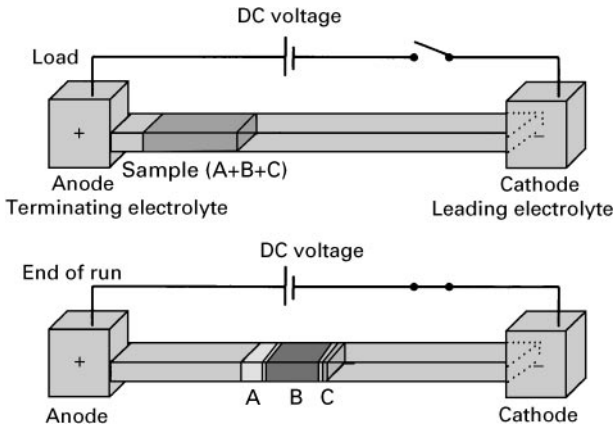


**Figure 7** (See Colour Plate 9) The principle of isotachophoresis.

using buffers of differing mobility at constant current is used. It is therefore a discontinuous system. Electrophoresis proceeds until all ionic analytes are migrating with the same (*iso*) speed (*tacho*) (**Figure 7**). The theory is dependent on the Kohlraush regulatory function (1897), which defines the conditions at the boundary between two different ions A and L such that

$$[A] = [L] \times \text{a constant}$$

For ITP to occur, an unbuffered sample should be placed between a leading electrolyte whose $\mu_{\text{buffer ion}} \gg \mu_{\text{sample}}$ and a terminating electrolyte with a $\mu_{\text{buffer ion}} \ll \mu_{\text{sample}}$. Leading electrolytes are usually small ions such as chloride and the terminating electrolytes are larger buffer ions such as histidine.

Applying the voltage causes the analytes to separate into zones according to their mobilities, but they must remain adjacent in order for the current to be carried. From Ohm's law all zones including the terminating and leading electrolytes must carry the same current so $R$ and $V$ must increase. When a steady-state is obtained all bands have the same ionic concentration because they must have the same $R$ so they will be of differing lengths. Zones must therefore increase in concentration to match the concentration of the leading electrolyte. The resultant output is usually observed as a series of steps in a conductivity trace in which the length of each band is proportional to the amount of analyte. Since analysts are usually happier looking at peaks, these traces are often differentiated in order to generate a profile with peaks so that the distance between the peaks can be measured. The development time is proportional to the amount of sample injected and to the differences in $\mu$ of the sample components.

LKB devoted considerable sums of money to develop a commercial ITP system (the Tachophore) in the late 1970s but this was a commercial failure. Commercial systems are still available from Japan (Shimadzu) and the Czech Republic (Villa Labco). Although ITP is little used today as an analytical tool, it is commonly encountered in many zone electrophoresis protocols when the loading of samples in solutions differing from the BGE is recommended. ITP will often occur transiently in the loading band leading to a concentration of some analytes at the front or rear of the loading band.

## Electrophoretic Separation Media

Electrophoresis can be performed in free solution, but thermal and diffusional mixing are usually too great to give satisfactory resolution of the components. It has long been recognized that it is usually necessary to use some type of static support in order to contain the background electrolyte, contain the sample and limit diffusion. The primary function of the stabilized support media is to limit the dispersion that results from convective disturbances. The stabilization should not change the mobility of the ions significantly provided that neither absorption onto the support occurs nor do large analytes become physically trapped.

Over the years a large number of supports have been used as stabilizing media, but relatively few satisfy all the criteria for the ideal support (**Figure 8**). These criteria are a reproducible structure, good mechanical strength, ease of formation into appropriate shapes, chemically inert to both the electrolyte and the analytes, no thermal degradation and minimal background in the detection processes. The earliest support was probably cellulose in the form of sheets of laboratory filter paper. Kunkel and Tiselius published the classic study on paper electrophoresis in 1951. Cellulose acetate membranes have large pores and do not retain proteins either chemically or physically. It is the preferred membrane for serum protein separations.

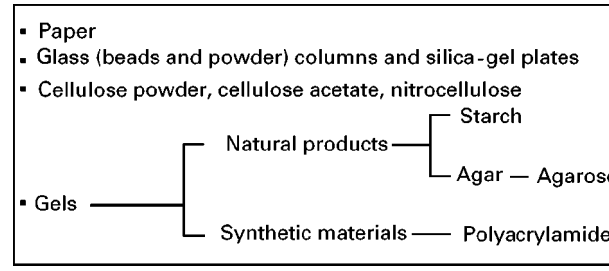


**Figure 8** Common stabilized media used in electrophoresis.

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## Slab Gel Electrophoresis

Subsequently naturally occurring gels were investigated particularly for the separation of plasma proteins. Agar gels, especially in the form of the highly purified subunit agarose, are still a popular medium for protein separations. Agar forms gels at relatively low concentrations, but due to its high number of sulfate and carboxylic groups it generates a significant EOF. The sulfate and carboxylic fraction can be removed chemically to leave agarose, which has much reduced EOF and gives a better resolution of proteins. The gels are formed as slabs by pouring a liquid gel into a mould and letting it set before placing in an electrophoresis system.

In 1955 Smithies, modifying an earlier method, introduced the technique of starch gel electrophoresis and its superior resolving power for proteins was immediately apparent. The starch was derived from hydrolysed potato starch and poured to a thickness of 5–10 mm. In addition, the pores in the starch gel matrix are close to the molecular size of proteins so that in this form electrophoretic movement is accompanied by some molecular sieving, but the effect was irreproducible.

In 1955 Raymond and Weintraub introduced a synthetic polymer gel made from the monomer polyacrylamide, as a replacement for starch. Polyacrylamide gels have many advantages over starch being tougher, more flexible, naturally clearer and chemically inert. Acrylamide gels are formed by the polymerization of the monomer acrylamide in the presence of an *N*,*N*′-methylenebisacrylamide (BIS). The reaction requires initiators and a catalyst (cross-linking reagent). Commonly used catalysts are ammonium or potassium persulfate (for chemical polymerization) or light and riboflavin (for photopolymerization). A common initiator is *N*,*N*,*N*′,*N*′-tetramethylethyldiamine (TEMED). The polymerization should be performed at above 20°C to prevent incomplete polymerization. The reaction takes place via vinyl polymerization and gives a randomly coiled gel structure. The concentration of polyacrylamide can be varied over a wide range without making the gels unmanageable. The pore size can also be controlled exactly by varying the amount of BIS used in the polymerization (**Table 3**) so the precision of the molecular sieving is enhanced.

The use of such gels for electrophoresis is commonly referred to as polyacrylamide gel electrophoresis (PAGE). In addition to the tight control of pore size other advantages of polyacrylamide gels compared to starch gels are that the adsorption of macromolecules to polyacrylamide is negligible, there is little EOF associated with polyacrylamide and strong, but thin, transparent gels can be cast permitting faster separation. A disadvantage is that the monomers are toxic and need to be handled with caution.

**Table 3** Effect of cross-linking on pore size of acylamide gels

| *Percentage of N,N′-methylenebisacrylamide (BIS)* | *Molecular weight range resolved* |
|---|---|
| 5 | 50 000–300 000 |
| 10 | 10 000–100 000 |
| 15 | ~ 5 000 |

Development of the gel system followed rapidly. In 1964 Ornstein and Davis simultaneously introduced discontinuous (DISC) electrophoresis, which improved both the solubility of the proteins in the gel as well as improving the resolution. In DISC electrophoresis the gel is formed in two sections, a stacking gel and a resolving gel. The resolving gel has small pores filled with a buffer of pH 8.8 high mobility buffer (e.g. 2-amino-2-hydroxymethylpropane-1,3-diol–hydrochloric acid (TRIS–HCl)) and a large pore stacking gel contains a buffer of about pH 6.8. The sample is loaded at approximately pH 8.8. These conditions induce the proteins to migrate according to isotachophoresis through the stacking gel, then stacked at the interface with the resolving gel before slowly destacking and resolving as they pass through that gel.

The size and shape of macromolecules complicates their separation, so special buffer/electrolyte conditions are employed. Non-dissociating (native) buffer systems, as described earlier for DISC electrophoresis, are used to separate the native forms of proteins and double-stranded DNA. Dissociating (denaturing) buffer systems can also be used, for example, double-stranded DNA is denatured using urea, formamide, sodium hydroxide or intercalating agents such as ethidium bromide prior to application to the gel.

Although it is appreciated that the use of gels not only aided the electrophoretic separation, for macromolecules it also introduced a size-sieving effect equivalent to gel filtration in chromatography. This can be used to 'size' molecules, particularly proteins. So if the conditions are correct and if the pores are of the appropriate dimensions could PAGE also be used to determine the molecular weight of proteins?

## Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Unlike DNA, proteins exhibit considerable differences in their overall charge-to-mass ratios.

Depending on pH, their charge can range from basic to anionic and from hydrophobic to hydrophillic, and their shapes can vary from globular to linear with varying degrees of cross-linking. Shapiro introduced electrophoresis in the presence of sodium dodecyl sulfate (SDS) which is an anionic surfactant that is used to solubilize proteins. Proteins are first denatured with SDS which binds to the hydrophobic backbone of proteins in a regular fashion. Almost regardless of the type of protein, 1.4 g of SDS binds to 1 g of protein and solublizes it. In addition, the charge on the SDS overwhelms the charges on the protein. The degree and type of denaturing can also be enhanced by using SDS solutions which also contain urea and/or a thiol such as dithiothreitol (DTT). The complex is now more or less linear with an overall negative charge so all proteins migrate in a single direction. The denatured unknown proteins are then electrophoresed by PAGE in a buffer system incorporating 0.1% SDS. By running a series of known standards in a parallel lane, the technique can be used to derive molecular weight information since there is a fixed relationship between the relative migration distance of SDS–protein complex and the molecular weight of the native protein.

## Pulsed Field for DNA

Agarose gel electrophoresis is the method of choice to resolve DNA restriction fragments provided the fragments are between 1000 and 23 000 bp in size. For larger fragments, Schwartz and Cantor developed the technique of pulsed field gel electrophoresis (PFG) in 1984. In PFG DNA fragments greater than 23 kbp are forced to change their structure during electrophoresis by pulsing the applied field so causing the molecules to relax and expand regularly and thereby interact with the gel pores. DNA molecules up to the size of chromosomes can be electrophoresed in this manner, but the separation can take many hours or even days.

## Basic Instrumentation for Electrophoresis

Instrumentation for performing electrophoresis is both cheap and simple. It consists of a means of generating a DC voltage, a buffer tank which also has some means of holding the separation media between the two electrodes. A schematic diagram of such a horizontal tank is shown in **Figure 9**. Such an apparatus needs to be levelled in order to prevent the electrolyte from siphoning from one end to the other and so disturbing the electrophoretic resolution. The

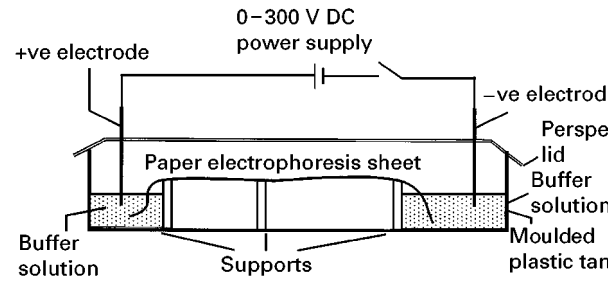


**Figure 9** Schematic diagram for a paper or membrane electrophoresis system.

power supply can be as simple as a 12 V battery, but is usually a mains electricity transformer delivering 100–500 V DC. The required voltage is selected by a digital potentiostat combined with a polarity switch, the run time can also be selected digitally. Usually there are controls to select constant voltage, constant current and constant power modes of operation. If the instrument's designed maxima in any of these modes are exceeded there is a cut-off (safety) trip. Electrical power is usually taken to the electrophoresis tank via a pair of suitably rated copper cables. The tank itself is typically made of a waterproof and electrically insulated plastic material covered with a lid to prevent evaporation and contamination of the system. The size of the tank will depend on the type of separation being undertaken and can range from as small as 100 mm square to as large as 500 mm × 300 mm. The tank is divisible into a number of compartments with plastic spaces. The outer two hold the electrodes. The electrodes are usually made of fine platinum wire connected through the tank wall to the leads from the power supply. Suitable safety devices are included in the tank to prevent operator accidents, for example the power is cut-off if the lid is removed.

With the development of gel electrophoresis system new forms of apparatus were developed. It is necessary to mount and support the gel between the two electrode chambers so that the only electrical connection is via the gel. Electrical contact between the gel and the electrolyte is made either directly or by a wick of some description. Gels can be of many sizes depending on the separation distance required. Tubes of gel with an internal diameter of 1–5 mm and 50–250 mm in length can be prepared, but slab gels, either used vertically or horizontally, are much more common. Vertical slab gels are cast by pouring liquid gel into the space between a pair of glass plates separated by thin spacer strips positioned at the edges and bottom. The plates are clamped to prevent leakage. The thickness of the gel depends on the thickness of the spacers, which can be from 50 μm to 5 mm

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depending on the application. At the top, a sample well comb is used to create indentations into which samples can be placed. Following the setting and/or polymerization of the gel, the comb is carefully removed and sample(s) (5–10 μL) are loaded into each well. Molecular weight markers, known proteins or DNA fragments, are usually applied to one or both of the two outer lanes. The upper and lower buffer chambers are then filled and the system is connected to the power supply. Appropriate voltages are selected and the power is turned on for the appropriate time. In order to monitor the progress of the separation it is common practice to include a marker dye in the sample. When the dye reaches the end of the gel (normally after some hours) the power is disconnected. The buffer is drained and the gel removed from between the plates prior to staining or blotting (**Figure 10**).

As well as vertical formats it is also possible to run horizontal slab gels either completely immersed in electrolyte solution (so-called submarine gels) or with suitable wetted connectors.

Although it is relatively easy to construct your own low-voltage electrophoresis system, especially the tanks, systems are available from a large number of suppliers, such as Amersham-Pharmacia, Bio-Rad, Hoeffer, and so on. The cost of commercial systems range from a few hundred pounds to about £5000 depending on size, configuration, maximum voltage and whether or not cooling systems are included. Today, even the preparation of gels can be simplified. Premixed reagents are available to simplify the production of reproducible gels and it is now possible to buy precast gels which only need to be mounted in a suitable holder.

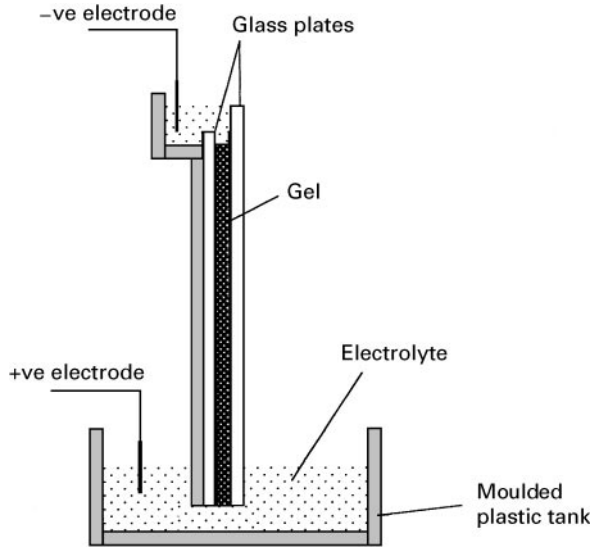


**Figure 10** Schematic diagram of a vertical gel electrophoresis system.

## Detection Methods in Electrophoresis

At the end of an electrophoretic run the analytes are distributed over a two-dimensional surface or included within a thin three-dimensional gel. With only a few exceptions, the majority of analytes will not be visible to the naked eye and many methods for both the detection, localization and quantitation of separated bands and spots have been developed.

Direct optical methods can be applied in some cases. For example, the bands of haemoglobin separated by electrophoresis on a nitrocellulose membrane can be observed directly. In some cases placing the paper or gel on a ultraviolet (UV) lightbox or under a UV lamp can reveal UV absorbing bands, UV fluorescence bands and sometimes bands that quench the background UV fluorescence.

Since the analytes are often trapped within a fragile gel, then some means of extracting them from that matrix often is necessary before a visualization method can be applied. For this purpose, one of the blotting techniques is applied. Blotting involves the transfer of the sample bands or spots from a gel to a sensitized membrane using either physical or electrical mechanisms. Nitrocellulose, Nylon or polyvinylidene difluoride (PVDF) are the most common membranes because they provide hydrophobic surfaces on which proteins readily adhere, as well as being physicaly strong. In 1975 Professor Ed Southern described the first of these procedures, which is commonly called Southern blotting, for use with DNA. In this method, diffusion of DNA from a continuously wetted electrophoresis gel to a membrane is achieved by pressure applied to a sandwich of wetted wick-gel-membrane–stack of blotting paper (**Figure 11**). The DNA in the gel is carried into the membrane that binds the DNA giving a faithful image of the original gel. The process takes up to 24 h but can be speeded by using a vacuum to draw the buffer through the gel.

The same procedure when applied to RNA is playfully, but consistently, called a Northern blot. In the same vein a Western blot is any procedure for the transfer of proteins from a gel to a membrane. Although with proteins this usually involves their electromigration linked to immunodetection with appropriate antibodies. Transfer using electrophoretic techniques applied across the thickness of the gel is generally faster than capillary action. The gel, containing protein or DNA, clamped next to the blotting membrane in a cassette, is then immersed in a buffer solution between two electrodes.

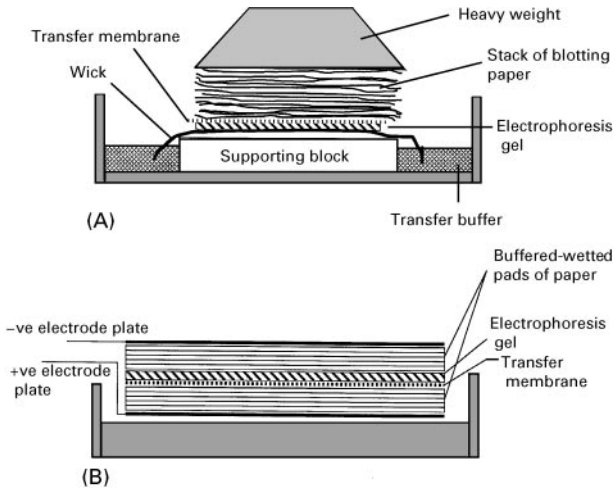


**Figure 11** The principles of two types of blotting: (A) Southern blotting and (B) semi-dry Western blotting.

Applying a voltage causes the proteins/DNA to migrate to the membrane. If filter papers wetted with buffer are used instead of buffer, then this is called semi-dry blotting. Once the analytes have been stabilized on the membrane they then can be probed in order to detect the analytes (this is discussed further below).

If separations were performed on sheets or have been blotted, then the position of the analytes can be determined by the following methods. If radioactivity has been incorporated in the analytes prior to electrophoresis, then the position of the analytes on the membrane can be determined by autoradiography. The membrane is placed with the gel in contact with photographic film and left to expose (hours to weeks) in the dark and possibly the cold. The film is developed and the position of the analytes is revealed by the exposed parts of the negative. If required, the original membrane or gel can then be overlaid on the negative and the radioactive spots obtained by cutting-up the gel followed by elution and scintillation counting.

It is often necessary to revel analytes of interest using some form of derivatization. This may mean spraying the blot or membrane with a specific reagent that chemically reacts with the analyte to give a coloured product, for example, ninhydrin gives a purple product with amino acids, peptides and some proteins. For macromolecules it is more common to refer to this process as staining using either appropriate dyes or other reagents. Proteins can be detected in gels with a large number of dyes of which the most common is Coomassie brilliant blue which can detect as little as 0.3 μg of protein in a spot. DNA is usually detected using the fluorescent intercalating dye ethidium bromide. The process involves staining followed by destaining to remove unbound dye and then fixing of the image. Both proteins and DNA can react with silver ions to form black silver precipitates. This method is some 100 times more sensitive than the current dye methods and can detect as little as 2 ng of some proteins.

The above methods are relatively non-selective, although for simple mixture comparisons against standards they may be sufficient to identify proteins. More specific methods of detection include zymography and immunoelectrophoresis. Early methods include immonodiffusion in which an antiserum is allowed to diffuse into a gel so generating precipitin bands and rocket electrophoresis. Today the most common application of immunotechniques is in Western blotting with immunodetection; this is so common that many workers believe it is Western blotting. After blotting, the immobilized proteins are probed with primary antibodies specific for the protein(s) of interest. This is followed by an enzyme-linked second antibody directed against the first antibody, for example sheep anti-mouse immunglobin G (IgG). The enzyme is usually a peroxidase or alkaline phosphatase. The blotted membrane is first soaked in a solution to block non-specific binding sites followed by incubation with a suitably diluted solution of the first antibody for up to 1 h. The antibody solution is then washed off before incubating with the secondary antibody complex. Again, following washing, the proteins are revealed by incubation with an appropriate visualization reagent. Depending on the antibodies available such methods can be very selective and also very sensitive.

Finally, the most modern methods of both detection and protein characterization involve mass sepectrometry and are dealt with below.

### Quantitation

The above methods are usually indirect and qualitative, so if you wish to obtain quantitative analytical data from electrophoretic separations or blots it is necessary to determine the area of the separated bands. The amount of analyte present is related to the area of the spot or band when compared to standard solutions. The areas of bands can be obtained using densitometry or digital image analysis to scan the gels or blots. However, even modern densitometry is only semi-quantitative with coefficients of variation of 10–20% at best.

### Two-dimensional Separations

Since traditional electrophoresis is usually planar, more than one separation dimension can easily be

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combined. The use of orthogonal techniques increases the resolution of the systems in proportion to the product of the resolution of each dimension assuming the two modes are truly orthogonal. For example, separations that each give 10 bands in a single direction/mode will theoretically give 100 spots if combined. The sacrifice is, of course, speed, it will take twice as long, and increases the number of manipulations involved.

Early work such as peptide mapping often used high-voltage electrophoresis in the first dimension and then chromatography in the second. For a decade or more this was the standard method for generating tryptic maps for studies on such areas as haemoglobin variant analysis. However, each sample would take nearly two days to run, a day for each dimension.

The most popular two-dimensional electrophoretic method is the combination of IEF with SDS–PAGE. O'Farrell and Klose independently introduced this system in 1975. In O'Farrell's original system isoelectric focusing in ampholytes was used in the first dimension and the resulting tube gel was then carefully attached to a slab gel followed by running in the SDS–PAGE mode. This method was able to separate up to 1000 protein spots from a protein extract of a tissue. However, the system required considerable expertise to use due to the variation in the IEF step; many workers found it irreproducible. With the development of immobilized IPGs the ease of operation of the IEF component was, as already discussed, greatly improved and relatively simple two-dimensional systems are now available commercially. For gels of 250 mm × 250 mm up to 3000–4000 protein spots can be revealed by silver staining from extracts of tissues such as liver. In this mode electrophoresis is probably the separation technique with the highest resolution available to the modern researcher.

Proteome analysis (proteomics) is a major new field of biomedical research. The proteome is defined as the total complement of proteins found within an organism, cell, tissue or biofluid. While the human genetic code will soon be known, its relationship to cell function will only be ascertained by advances within proteomics. For example, DNA sequences are not predictive of post-translational modifications such as glycosylation within the encoded proteins. Proteomics is the method of qualitatively determining this large number of proteins. It combines the high-resolution two-dimensional electrophoretic techniques mentioned above with digital image analysis, some elements of HPLC and modern mass spectrometry plus on-line bioinformatics to search databases. For example, two-dimensional proteome maps can be produced for a tissue such as liver and then digitally compared with the same tissue taken from a diseased subject. The differences in the complex pattern, which may contain 1000+ spots, can be highlighted automatically and then mass spectrometry either of the MALDI-ToF and/or electrospray variety can be used to identify the variant proteins. By using such approaches it is now possible to identify known proteins using bioinformatic tools over the Internet in a matter of minutes, and to determine the structure of an unknown protein may only take a day or two.

### Fields of Application

Although almost every class of molecule, especially those of a biochemical nature, has been separated by planar electrophoretic methods few are now done so. Early textbooks on electrophoresis detail separations of amino acids, sugars, purines and pyrimidines, nucleotides, some drugs, carboxylic acids, vitamins, and so on, but today's analyst would now use HPLC or capillary electrophoresis for such quantitative studies. Only for protein and DNA molecules is planar electrophoresis still the method of choice? Electrophoretic methods are used for qualitative and quantitative separation of proteins in clinical samples, biotechnological production and quality control, genomic and proteomic studies.

### Traditional Planar Electrophoresis – Conclusions

This is still a basic tool in biochemistry and biomedicine since it is simple and often very cheap, parallel analyses are usual and so although any one run is slow, the many bands can be run at the same time so overall throughput is reasonably high. With the appropriate detection techniques, selectivity and sensitivity can be very good. By using thick media electrophoresis the technique can be made at least semi-preparative. Finally, in two-dimensional formats (IEF–SDS–PAGE) electrophoresis probably gives the highest resolution known in separation science.

However, perceived disadvantages are that it offers only low resolution in one-dimensional separations and the results are at best only semi-quantitative. Since it is difficult to use high voltages, it is seen as a slow technique made slower by the fact that detection is nearly always off-line.

Nevertheless there is a wealth of experience with the hundreds of available methods and probably millions of analytes are separated every day using traditional electrophoretic methods. Many of these analyses go unnoticed, for example the routine examination of serum proteins in clinical biochemistry laboratories throughout the world and the hundreds of daily runs sequencing DNA and so on in molecular

biology laboratories. However, research still continues into the technology and ultra-thin gels for DNA sequencing are giving excellent results and automated equipment for use with proteomics both in the electrophoresis stage and the sample isolation stage is being developed. Even after 100 and more years there is still much life in this technology.

## Capillary Electrophoresis (CE)

For 40 years it had been appreciated that the excessive Joule heating that developed when high voltages were used to speed-up electrophoresis caused many of the technique's disadvantages. These disadvantages ranged from simply boiling the buffer solutions through to instrument fires and even electrocution. Many attempts were made to overcome the physicochemical problems that degraded the separations. For a time in the 1960s and 1970s high-voltage electrophoresis systems operating at up to 10 kV were available. They were usually very large pieces of equipment that used large sheets of chromatography paper for the separations and required substantial pumped water cooling systems to remove the heat generated. Other equally complex systems with rotating Pyrex tubes to equalize the heating effects were also developed.

As with many problems, the solution when it came was very simple. In 1981 Jörgenson rationalized the problem to one of increasing the rate of cooling by substantially increasing the surface-to-volume ratio of the electrophoresis buffer. This, he showed, was readily achieved by performing the electrophoresis in small-bore capillaries. In a classic series of papers in that year Jorgenson and Lukacs described spectacular separations of peptides using zone electrophoresis in glass capillaries of 75 μm internal diameter using voltages of up to 30 kV with electrokinetic injection and fluorescence detection. This format enabled the Joule heating that normally degrades the resolution by thermal mixing to be efficiently dissipated. In addition, this new format used the significant electroosmotic (EOF) flow of the background electrolyte in the capillaries to separate cation, anions and uncharged molecules simultaneously with all analytes usually going towards the cathode where a single on-line detector was placed.

This discovery led to the development of a new form of analytical separation instruments that was very equivalent in its operation to HPLC. Most of the major HPLC instrument manufacturers had by the end of the 1980s introduced CE systems with varying degrees of sophistication. There was also considerable optimism that CE was going to replace HPLC for analytical separations. Ten years later only three companies are still marketing complete CE instruments.

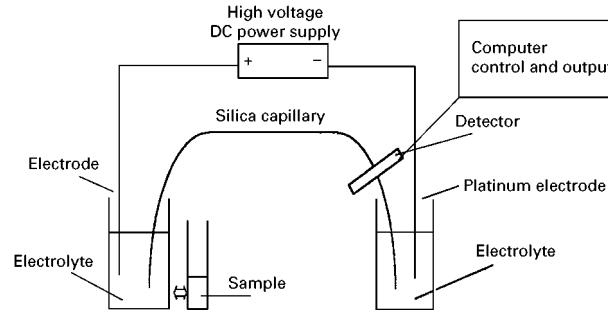


**Figure 12** The configuration of a simple capillary electrophoresis (CE) instrument.

Today CE is characterized by its ability to resolve, using applied DC voltages giving field strengths up to 500 $V^{-1}\,cm^{-1}$, the components of complex aqueous samples with very high resolution ($N > 250\,000$) analysing less than 10 nL of sample with analytical precision. To maintain efficiency, detection (UV, fluorescence) is nearly always on-line, i.e. across a window burnt into the polyimide coating of a silica capillary. Detection at the end of capillary can be achieved using electrochemical detectors or more usefully mass spectrometry.

A typical CE instrument consists of a capillary, detector, high-voltage power supply, recording device (**Figure 12**). The capillary used is made of fused silica and externally coated with a thin layer of polyimide, to make the capillary flexible. The capillary is usually about 375 μm outside diameter and 10–150 μm inside diameter with 50 and 75 μm the more usual. There is no set capillary length, although in commercial instruments there are minimum lengths which can be used, and this is usually dependent on the position of the detector. To allow detection a small section of polyimide coating has to be removed making a weak point in the capillary.

In CE electroosmotic flow (EOF) is a positive benefit, unlike in traditional electrophoresis systems. The silanol groups on the inner surface of the silica capillary are negatively charged above approximately pH 3 so cations build up near the silanol groups to maintain the charge balance. When the potential difference is applied to the capillary these cations are attracted towards the cathode, and they drag the bulk solution along with them (**Figure 13**).

The magnitude of the EOF is dependent on the surface charge of the capillary silanol groups, which varies with pH so that the EOF velocity increases with increasing pH. The flow profile of the EOF is different to the solvent flow profile found in HPLC. The laminar or parabolic flow produced by

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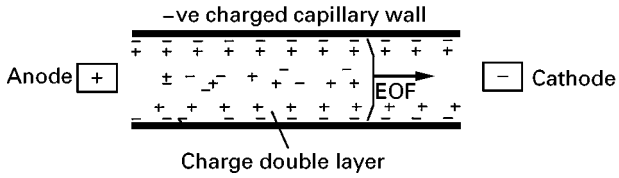


**Figure 13** The electroosmotic flow (EOF) profile in a silica capillary electrophoresis (CE) capillary.

a liquid chromatography pump is due to the shear force at the capillary walls. In CE there is no pressure drop and therefore a flat flow profile is produced giving very much sharper peaks. In addition, at pH > 6 the EOF will be of sufficient magnitude to carry all the analytes regardless of their change in the same direction, this will only happen if the magnitude of the EOF is greater than the electrophoretic mobilities of the anions. The EOF will also carry neutral molecules along, although it cannot separate them. Since everything is moving in one direction an on-line detector can be placed at the cathodic end of the capillary. The output from a CE system is therefore very similar to that from HPLC systems – a series of peaks with baselines drawn in (if required) and areas of peaks calculated.

The present generation of commercial CE instruments is capable of unattended operation and features auto-injection using a variety of modes, integration and spectral analysis in a manner analogous to HPLC. CE has become widely available and the technology is now included in a number of other instruments such as a clinical protein analyser (Beckman Paragon) and in a DNA sequencer (PE-ABI 3700). In both of these instruments a large number of capillaries are operated in parallel in order to increase sample throughput, but the end users buys an instrument dedicated to a specific application not a CE instrument.

**Modes of operation for CE** CE is generally recognized as the description appropriate for the whole field of this separation science. CZE stands for capillary zone electrophoresis, in which ions are separated according to their mobility in free solution. This is the most frequently used separation mode option available within CE. A subtype of CZE is capillary ion analysis (CIA), which is used to determine simple ion species in aqueous solution rapidly. The other forms of electrophoresis described earlier such as isoelectric focusing and isotachophoresis can also be performed in a capillary format and are called cIEF and cITP, respectively. MEKC, sometimes called MECC – micellar electrokinetic capillary chromatography – is a separation mode introduced in 1984 by Terabe which allows the separation of neutral molecules by their differential partition into charged micelles formed from detergents incorporated into the CE electrolyte. MECC can also be viewed as a subclass of CE methods involving complexation and inclusion using additives to the background electrolyte. Another separation mode is capillary gel electrophoresis (CGE), which was introduced by Cohen and Karger in 1988. This offers DNA and SDS–PAGE separations in a capillary, but with advantages in terms of speed, quantitation and ease of automation. The filling of capillaries with gels has now been supplanted by the use of entangled viscous polymer solutions to separate DNA. A recent development is the use of EOF to drive eluent through a capillary packed with HPLC stationary phase. This is termed capillary electrochromatography (CEC) and some very high efficiencies for neutral compounds have been demonstrated using this technique. CEC is performed on standard CE instrumentation, but has the added advantage that the eluents used are more readily interfaced to MS than is CE.

There has been a great deal of debate about the benefits and disadvantages of CE and it is worth touching on a few points here. The immediate attraction of CE for many analyses is its high efficiency giving the resolution needed to analyse complex samples and mixtures of biomolecules. CE can readily achieve separation efficiencies of orders of magnitude greater than those obtained by HPLC. From 100 000 to 250 000 theoretical plates per capillary is common, while up to 30 million have been reported for the CGE of oligonucleotides. Such efficiencies mean that 'isocratic' CE is capable of improving on the resolution obtained by both isocratic and gradient HPLC. **Figure 14** shows the separation of the complex array of metabolites and exogenous compounds excreted in normal human urine. A simple MEKC separation is shown resolving over 60 peaks in about 10 min. CE separations are simpler both in operation and equipment, and potentially much faster and quantitative. Thirty per cent more peaks have been observed resolved by CE compared to gradient HPLC under similar sample detection conditions. By using a high potential in short, narrow capillaries very fast assays using CE are possible. Many small ions can be resolved in only a few minutes. So-called short-end injection is also useful for high-speed separations. Such runs combined with minimal sample preparation means that some assays can take less than 1 min by CE. Mass sensitivity in CE is superb, as proved in the many elegant experiments that have determined various components in a single living cell. However, the concentration sensitivity is about one order of magnitude lower than in HPLC for the same detector. This is because the probed volumes

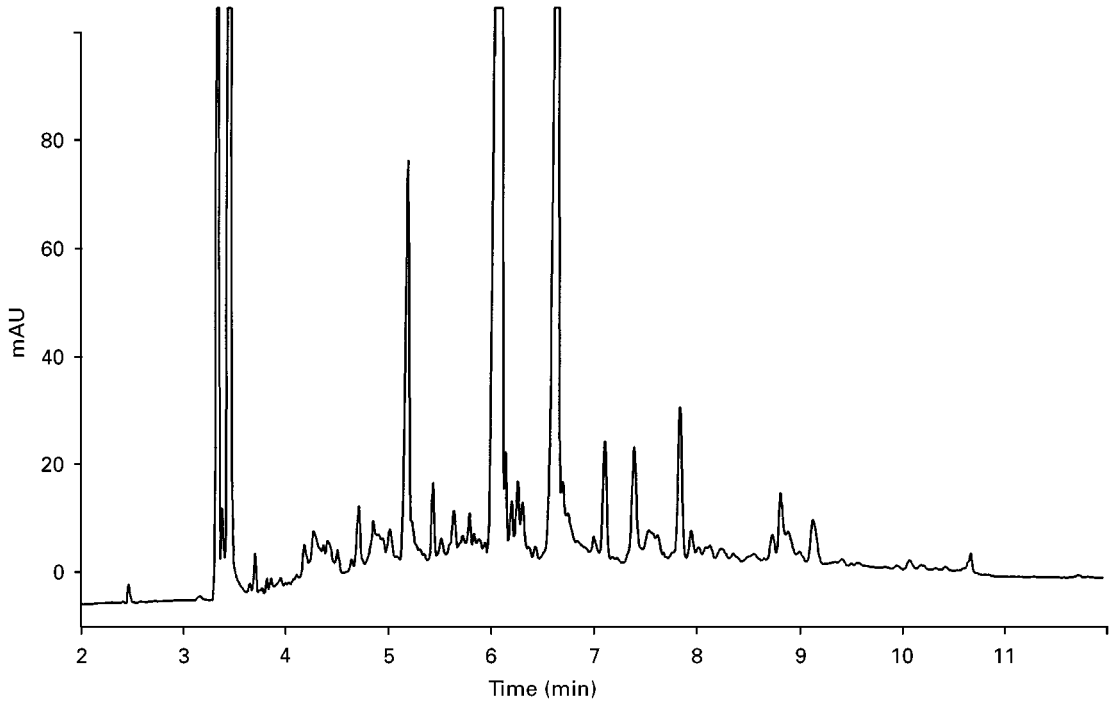


**Figure 14** High-resolution separation of UV absorbing components of human urine separated by MEKC with detection at 195 nm.

are correspondingly lower, e.g. UV detection samples across a 50 μm capillary in CE compared to 10 mm in HPLC. There is much activity in detector development to address the problem of sensitivity. Laser-induced fluorescence (LIF) has been studied widely and promises to bring detection limits down to the single molecule level for appropriate fluorophores. LIF detection is now commercially available. CE-MS with electrospray ionization has proved to be a powerful technique with applications to structural studies of large biomolecules as well as small ions, and there is good concentration sensitivity using capillary isotachophoresis as the separation mode.

**Applications** Clearly CE is very good for separating charged species whether small molecules or macromolecules. It is necessary to operate in MECC mode to separate neutral molecules such as drugs. There are now some 5000 publications describing separations of some 10 000 compounds by CE. Charged and neutral compounds are all easily separated but there are too many examples in the literature to even attempt to describe them here. The high resolving power of CE is such that it can readily resolve enantiomeric compounds. The most commonly employed method is to include cyclodextrins into the electrolyte. $\beta$-Cyclodextrin is the most readily available compound, but recently sulfated derivatives of $\beta$-cyclodextrin have been shown to give separation of chiral compounds with spectacular resolution between the enantomers. Oligonucleotides have proved to be readily separated using entangled polymeric additives such as hydroxyethylcellulose in the electrolyte to increase its viscosity and therefore gel-filtration effects. While the potential for quantitation shows enormous promise for proteins, there are several problems which remain to be overcome. Wall interactions degrade resolution and at worst lead to total adhesion of proteins to negatively charged sites on the silica capillary. Strategies to minimize this include covalent bonding to give coverage of the surface by hydrophobic groups, and dynamic coating using solution-phase additives.

In comparison to HPLC, CE achieves better resolution than both isocratic and gradient HPLC using simpler instrumentation. CE is not a preparative technique, although it has been used as a micropreparative system to isolate very small amounts of protein for sequencing. It is less sensitive than HPLC by about an order of magnitude. There is little difference in terms of quantitative data and analytical precision. Sample preparation probably needs to be better controlled and understood than for HPLC. At present, CE instrumentation is more expensive than HPLC, although running costs are considerably lower. CE uses much less sample and reagents than HPLC. Waste disposal problems are considerably reduced.

CE has now found a role in many laboratories and offers useful complementary separations to HPLC. It is clearly an established technique with a long-term future in the separation sciences.

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**See Colour Plates 6, 7, 8, 9.**

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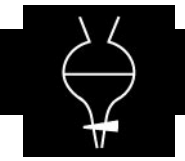
# EXTRACTION



**D. E. Raynie**, The Procter & Gamble Company, Cincinnati, OH, USA

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## Introduction

Extractions are common in the world around us. Each time we brew a cup of tea or a pot of coffee, and each time we launder our clothes, we're performing a chemical extraction process. Perhaps because of this familiarity, extraction processes in chemical laboratories are often not fully appreciated, or fully understood. Quite simply, an extraction is the process of moving one on more compounds from one phase to another. Yet behind this simple definition lies a great deal of subtlety: separations are contrary to thermodynamic intuition, because entropy is gained through mixing, not separation; extraction methods are developed based on a drive towards equilibrium, yet the kinetics of mass transfer cannot be ignored. Such a list of physical chemical nuances provides the basis for this chapter on the fundamentals of chemical extractions.

Extractions are carried out for a variety of reasons, for example when distillation is either impractical (e.g., distillations are favourable when the relative volatility of the compounds to be separated is greater than about 1.2) or is too expensive, to isolate material for characterization, to purify compounds for subsequent processing, etc. Extractions can be classified according to a number of schemes:

- analytical versus preparative (depending on the quantity of pure compound to be separated);
- batch versus continuous (depending on the mode of feeding the material to be separated into the extraction apparatus);
- based on the physical principles involved (is the extraction strictly based on partitioning, or are adsorption or other processes involved?);
- based on the types of phases involved (so called liquid–liquid extraction, gas–solid extraction, supercritical fluid extraction, etc.).

Perhaps the biggest recent advances in the field of chemical extractions have taken place in the petroleum, nuclear, and pharmaceutical industries. The understanding and practise of extraction lies at the crossroads of analytical, inorganic, organic, and physical chemistry, with theoretical and applied chemical engineering. Yet the fundamental physicochemical principles involved are the same. Because of the author's background, this chapter presents a description of the fundamental basis for chemical extractions and an overview of extraction techniques with a slant, or emphasis, towards the analytical chemists' perspective.

In general, the extraction process occurs as a series of steps. First the extracting phase is brought into intimate contact with the sample phase, usually by a diffusion process. Then the compound of interest partitions into or is solubilized by the extracting solvent. With liquid samples this step is generally not problematic. However with solid samples, for the compound being extracted to go into the extracting solvent the energy of interaction between the compound of interest and the sample substrate must be overcome. That is, the material's affinity for the extracting solvent must be greater than its affinity